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Development of novel oxazolo[5,4-*d*]pyrimidines as competitive CB₂ neutral antagonists [1] based on scaffold hopping

Wei Tuo,^a Mélanie Bollier,^a Natascha Leleu-Chavain,^a Lucas Lemaire,^a Amélie Barczyk,^a Xavier Dezitter,^a Frédérique Klupsch,^a Fabien Szczepanski,^a John Spencer,^b Philippe Chavatte,^a and Régis Millet^{a,*}

^a ICPAL, Univ. Lille, Inserm, U995-LIRIC-Lille Inflammation Research International Center, 3 Rue du Professeur Laguesse, BP83, F-59006 Lille, France

^b Department of Chemistry, School of Life Sciences, University of Sussex, Falmer, Brighton, East Sussex BN1 9QJ, U.K.

Abstract: A series of novel oxazolo[5,4-*d*]pyrimidines was designed via a scaffold hopping strategy and synthesized through a newly developed approach. All these compounds were evaluated for their biological activity toward CB₁/CB₂ cannabinoid receptors, their metabolic stability in mice liver microsomes and their cytotoxicity against several cell lines. Eight compounds have been identified as CB₂ ligands with K_i values less than 1 μ M. It is noteworthy that 2-(2-chlorophenyl)-5-methyl-7-(4-methylpiperazin-1-yl) oxazolo[5,4-*d*]pyrimidine **47**

Abbreviations: Boc, *tert*-butoxycarbonyl; cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary cells; CHO-CB₂, Chinese hamster ovary cells expressing CB₂ receptors; CHO-WT, Chinese hamster ovary cells wild type; DIEA, *N,N*-diisopropylethylamine; ECS, endocannabinoid system; KHMDS, potassium bis(trimethylsilyl)amide; SAR, structure-activity relationship; TFA, trifluoroacetic acid.

* Corresponding author.

E-mail address: regis.millet@univ-lille2.fr (R. Millet).

and 2-(2-chlorophenyl)-7-(4-ethylpiperazin-1-yl)- 5-methyloxazolo[5,4-*d*]pyrimidine **48** showed CB₂ binding affinity in the nanomolar range and significant selectivity over CB₁ receptors. Interestingly, functionality studies imply that they behave as competitive neutral antagonists. Moreover, all tested compounds are devoid of cytotoxicity toward several cell lines, including Chinese hamster ovary cells (CHO) and human colorectal adenocarcinoma cells HT29.

Key words: Cannabinoid, CB₂ receptor, neutral antagonist, oxazolo[5,4-*d*]pyrimidine

1. Introduction

The endocannabinoid system (ECS), present in mammals and comprising two primary G protein-coupled receptors, is linked with endogenous lipid mediators and relevant enzymes responsible for the synthesis and degradation of lipid mediators [2]. Due to its functions in the regulation of pain, inflammation, motor behavior, emotion, appetite and addiction, the ECS has been regarded as an ideal target with broad therapeutic potential [2-4]. The two G protein-coupled cannabinoid CB₁ and CB₂ receptors, are predominantly expressed in the central nervous system (e.g. brain) and immune cells (e.g. macrophages), respectively [3, 5-7]. CB₁ receptor antagonists have shown potential in the treatment of addiction and obesity. It has been demonstrated that the administration of a CB₁ antagonist (e.g., rimonabant) brings about a reduction of drug-seeking behaviors and weight loss [8-11]. By contrast, CB₂ receptor agonists lead to down regulation of inflammation and pain by restoring the equilibrium between pro- and anti-inflammatory cytokines [3, 5, 7, 12]. Moreover, several studies

have shown that CB₂ inverse agonists can regulate bone proliferation and relieve inflammation through modulating the migration of immune cells [13-15]. However, research on CB₂ neutral antagonists is rare, and their therapeutic potential remains unknown. In some cases, CB₂ neutral antagonists can be considered as ideal tools for investigating the biological function of receptors due to their ability to combine with receptors without altering corresponding biomolecular levels [16, 17]. In addition, targeting CB₁ receptors rather than CB₂ receptors has been demonstrated to induce tetrahydrocannabinol-like side effects (e.g., hypomotility, hypothermia, catalepsy) [18]. Thus, the development of selective CB₂ ligands has been considered as an interesting therapeutic strategy.

Over the course of the last few decades, the development of CB₂ ligands has significantly progressed. Numerous selective CB₂ ligands have shown therapeutic potential in a wide range of *in vivo* models, including pain, inflammation, cancer, immune disorders, neurodegenerative diseases and bone disorders [14, 19-26]. Specifically, more than ten CB₂ selective agonists have been reported to be under evaluation in clinical trials for the treatment of inflammation and pain [12]. For instance, compound **1** (GRC10693, Figure 1), developed by Glenmark Pharmaceuticals, successfully completed Phase I trials in 2009 [27]. It was intended to be used for pain remission, including neuropathic pain and inflammatory pain. Moreover, Eli Lilly and Co. disclosed a series of purines as selective CB₂ agonists [28-31]. Of note, compound **2** (LY2828360, Figure 1), derived from the lead compound **3** (Figure 1), has been recognized as a potent and efficacious analgesic

agent in a rat model of osteoarthritis related chronic pain at a 1 mg/kg oral dose [30]. Accordingly, clinical trials for the treatment of osteoarthritic knee pain were performed [32]. Additionally, it was reported that compound **4** (AM630, Figure 2), one of the most potent CB₂ inverse agonist identified to date, can attenuate osteoporosis [25, 33, 34]. Two other notable CB₂ inverse agonists, **5** (Sch225336, Figure 2) and **6** (Sch414319, Figure 2), have been shown to reduce inflammation through modulating the recruitment of immune cells [13, 15].

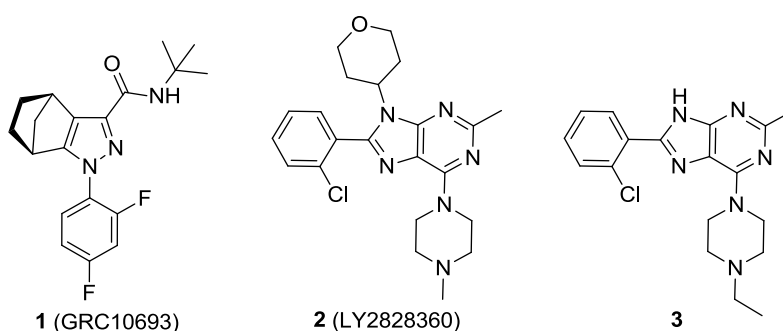


Figure 1. Structures of representative CB₂ agonists evaluated in clinical trials.

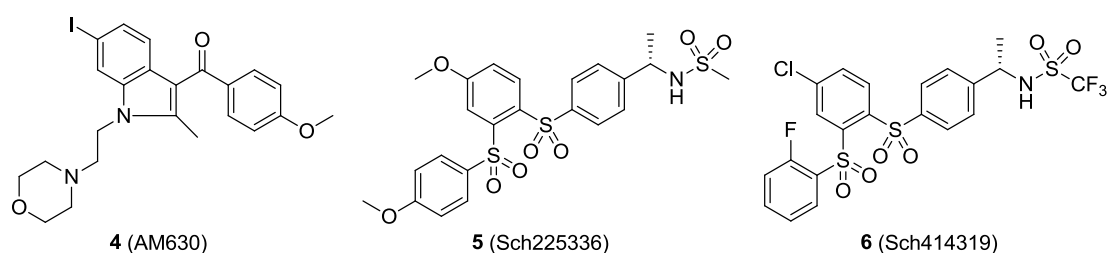


Figure 2. Representative CB₂ inverse agonists.

Scaffold hopping is a useful and powerful strategy for drug discovery. It has been widely used for the design of potential bioactive molecules [35-37]. In general, scaffold hopping incorporate heterocycle replacement, ring opening and closure, pseudopeptides and peptidomimetics, and topology/shape-based scaffold hopping [36].

Herein, we applied the heterocycle replacement-based scaffold hopping strategy toward compound **3**, a CB₂ agonist developed by Eli Lilly and Co [30]. Oxazolo[5,4-*d*]pyrimidine is a versatile scaffold used for the design of bioactive ligands against enzymes or receptors [38-40]. Hence, we replaced the purine core by this scaffold, with the intention of observing its influence on bioactivity toward CB₂ receptors (Figure 3). Afterwards, we carried out pharmacomodulations around the oxazolopyrimidine core (Figure 4). The newly synthesized compounds were tested for their CB₂ vs CB₁ affinity in a binding assay, their efficacy toward CB₂, and their metabolic stability in mice liver microsomes.

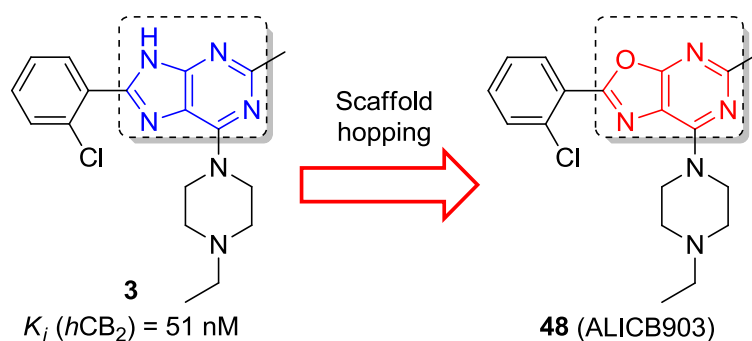
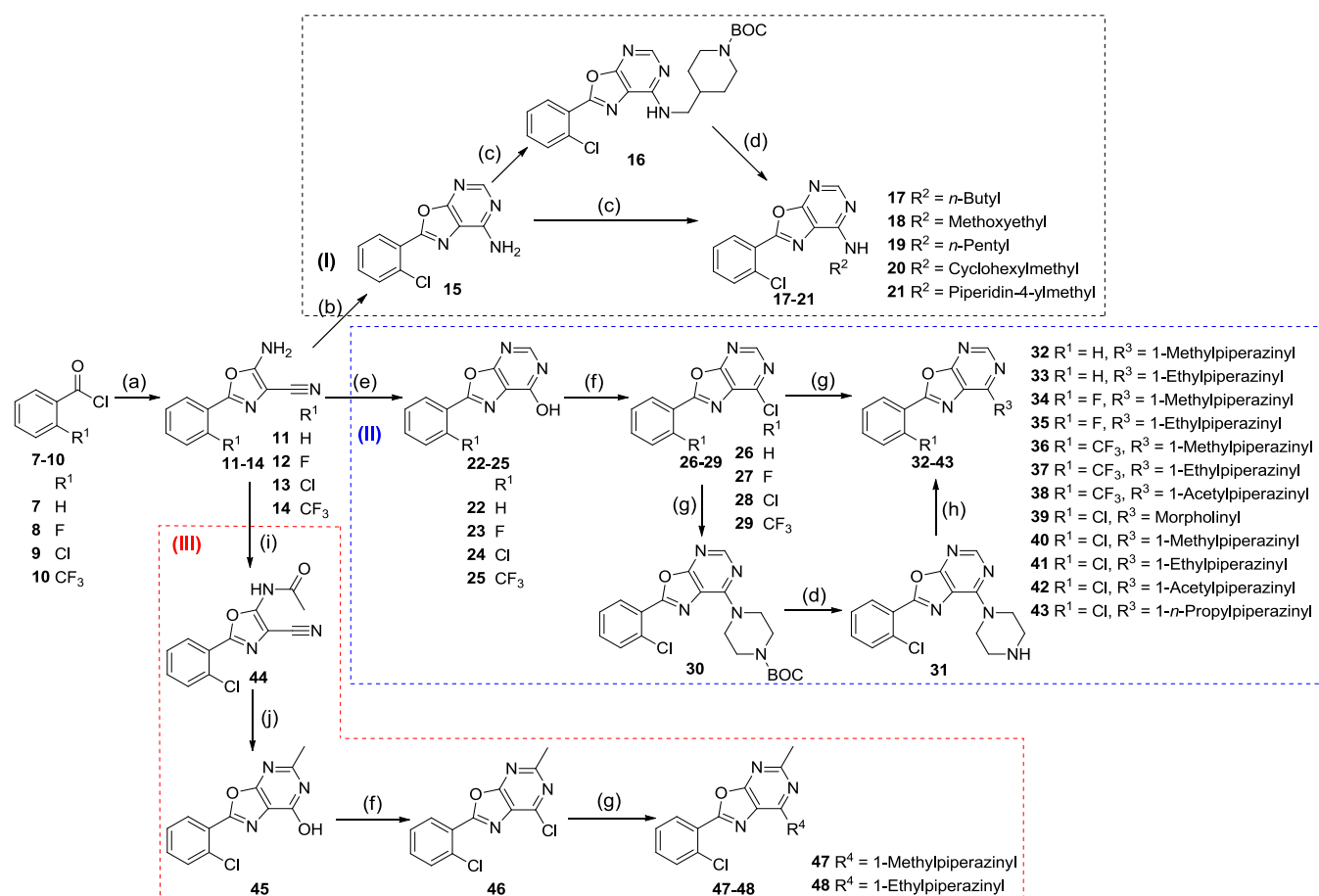


Figure 3. Heterocycle replacement-based scaffold hopping toward compound **3**.

afford a *tert*-butoxycarbonyl (Boc)-protected precursor **16** (43% yield). Deprotection of the Boc group of **16** using trifluoroacetic acid (TFA) followed by neutralisation yielded compound **21** (99% yield). (II) Alternatively, compounds **11-14** were cyclized using formic acid to give 2-phenyloxazolo[5,4-*d*]pyrimidin-7-ones **22-25** (33-46% yields), respectively. Afterwards, POCl₃-dependent chlorination converted these 2-phenyloxazolo[5,4-*d*]pyrimidin-7-ones into compounds **26-29** (72-85% yields). The chloride group of **26-29** was substituted by a piperazine or morpholine derivative to either give target compounds **32-42** (98-99% yields) directly, or to afford Boc-protected compound **30** (98% yield). Compound **43** (98% yield) was obtained after deprotection of the piperazine group of compound **30** using TFA followed by a nucleophilic substitution on bromopropane. (III) Additionally, compound **13** was acetylated to afford **44** (78% yield) in the presence of acetyl chloride and *N,N*-diisopropylethylamine (DIPEA). A solution of **44** in 30% NaOH and 35% H₂O₂ was refluxed to give **45** (69% yield), which was further chlorinated by POCl₃ (77% yield) and substituted with piperazine derivatives to produce compounds **47-48** (98-99% yields).



Scheme 1. Synthetic routes to prepare compounds **17-21**, **32-43**, and **47-48**. (a) aminomalononitrile p-toluenesulfonate, NMP, microwave, 80 °C, 25 min, 75-90%. (b) formamidine acetate, NMP, 100 °C, 3 h, 86%. (c) KHMDS, 15 wt. % in toluene, brominated derivative, DMF, room temperature (rt), 3 h, 37-52%. (d) TFA, CH₂Cl₂, rt, 3 h, 99%. (e) formic acid, reflux, overnight, 31-37%. (f) POCl₃, DIPEA, toluene, rt, 2 h, 72-85%. (g) piperazine derivative or morpholine, Cs₂CO₃, DMF, 100 °C, 30 min, 98-99%. (h) bromopropane, Cs₂CO₃, DMF, 100 °C, 30 min, 99%. (i) acetyl chloride, DIPEA, NMP, 45 °C, 6 h, 78%. (j) 30% NaOH and 35% H₂O₂ in water, reflux, 2 h, 61%.

2.2. *In vitro* binding assays and cytotoxicity

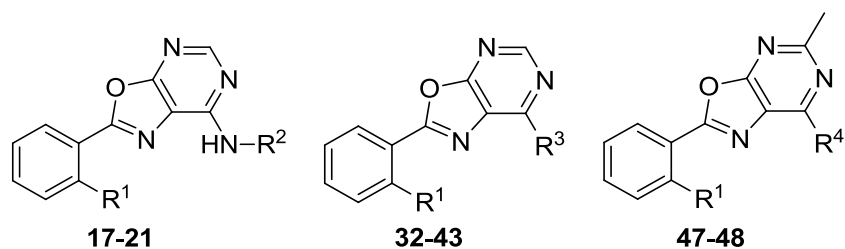
The affinities of each synthesized compound for CB receptors were determined

by a competitive radioligand displacement assay using the dual CB₁/CB₂ ligand [³H]-CP55,940 [22, 41]. All compounds were first screened at the concentration of 1 μM. The binding affinities (K_i) were calculated using the Cheng-Prusoff equation based on the experimental IC₅₀ values for compounds exhibiting a competitive binding superior to 50% for CB₂. K_d values of CP-55,940 towards CB₁ and CB₂ are 0.62 and 0.85 nM, respectively.

As illustrated in Table 1, compounds **47** and **48** bearing a chlorine at the ortho position of the phenyl ring, a C5-substituted methyl group, and a C7-substituted piperazine moiety, possess the best affinities toward CB₂ receptors, in the double-digit nanomolar range, with good selectivity over CB₁ receptors (SI > 12). The removal of the C5-substituted methyl group of **47** and **48** (K_i = 27.5 nM and 23 nM, respectively) leads to a sharp decrease in CB₂ binding affinities (i.e. compounds **40** and **41**, K_i = 350 and 329 nM, respectively). These results imply that the methyl group at the C5 position plays a crucial role in binding affinity for CB₂, probably due to hydrophobic interactions with the receptor. In general, C7-substituted piperazine derivatives (**40-42**, K_i values range from 350 nM to 678 nM) are preferred over morpholine (**39**, K_i > 1000 nM) or secondary amine substituents (**17-21**, K_i > 1000 nM). Of note, compounds bearing an *N*-ethylpiperazine at the C7 position (**37**, **41**, and **48**, K_i = 95.5 nM, 329 nM, and 23 nM, respectively) possess slightly superior CB₂ binding affinities than the corresponding compounds bearing a *N*-methylpiperazine (**36**, **40**, and **47**, K_i = 155 nM, 350 nM, and 27.5 nM, respectively) and are approximately 2-3 times more potent CB₂ ligands than compounds bearing a *N*-acetylpiperazine substituent (**38** and

42, $K_i = 254$ nM and 678 nM, respectively). Specifically, extending the carbon chain on the nitrogen at the C7 position of **41** ($K_i = 329$ nM) from 2C to 3C (**43**, $K_i > 1000$ nM) leads to almost complete loss of CB₂ binding affinity. Moreover, large *ortho* substituents (such as CF₃, **36-38**, K_i values range from 95.5 nM to 254 nM, and Cl, **40-42**, K_i values range from 329 nM to 678 nM) on the phenyl ring contribute to better CB₂ binding affinities in comparison with small groups (such as F, **34-35**, $K_i > 1000$ nM, and H, **32-33**, $K_i > 1000$ nM). This observation might be attributed to the fact that larger groups make stronger hydrophobic interactions with the CB₂ receptor.

Table 1. Affinities (K_i values) of compounds **17-21**, **32-43**, **47-48**, and reference compounds **3**, **4**, WIN 55,212-2 toward *h*CB₂ and *h*CB₁ cannabinoid receptors, selectivity ratios *h*CB₂ versus *h*CB₁, and cytotoxicity on CHO-WT, CHO-CB₂, and HT29 Cells.



Compounds	R ¹	R ²	Binding assays (nM) ^a		CB ₁ /CB ₂ ratio	Cytotoxicity assays % inhibition at 10 μM		
			K_i <i>h</i> CB ₂	K_i <i>h</i> CB ₁		CHO-WT	CHO-CB ₂	HT29
17	Cl		> 1000	> 1000	N.D. ^b	0	18	0
18	Cl		> 1000	> 1000	N.D. ^b	0	0	0
19	Cl		> 1000	> 1000	N.D. ^b	6	13	10
20	Cl		> 1000	> 1000	N.D. ^b	0	10	0
21	Cl		> 1000	> 1000	N.D. ^b	0	9	41
	R¹	R³						
32	H		> 1000	> 1000	N.D. ^b	0	0	0
33	H		> 1000	> 1000	N.D. ^b	0	0	0

34	F		> 1000	> 1000	N.D. ^b	8	7	0
35	F		> 1000	> 1000	N.D. ^b	10	8	24
36	CF ₃		155 ± 8	> 1000	> 6.4	3	0	0
37	CF ₃		95.5 ± 11.5	> 1000	> 10.4	10	5	1
38	CF ₃		254 ± 26	> 1000	> 3.9	12	1	12
39	Cl		> 1000	> 1000	N.D. ^b	0	10	5
40	Cl		350 ± 33	> 1000	> 2.8	0	0	8
41	Cl		329 ± 61	> 1000	> 3.0	1	9	4
42	Cl		678 ± 84	> 1000	> 1.4	23	11	0
43	Cl		> 1000	> 1000	N.D. ^b	0	2	0
	R¹	R⁴						
47	Cl		27.5 ± 0.5	> 1000	> 36.3	0	4	24
48	Cl		23 ± 4	293	12.7	0	16	16
3 (agonist)			51 ± 9	> 1000	> 19.6	30	0	0
4 (inverse agonist)			91 ± 11	> 1000	> 11.0	0	0	0
WIN55212-2			3.01 ± 0.99					
(agonist)								

^a Data represent the mean ± SEM of at least three experiments performed in duplicate or triplicate.

^b N.D. means not determined.

In summary, the structure-activity relationship (SAR) studies indicate that piperazine moieties at the C7 position favor CB₂ binding affinities of the molecules. Notably, lipophilic piperazine moieties (i.e., methylpiperazine and ethylpiperazine) are preferred for CB₂ affinity rather than a hydrophilic piperazine moiety (i.e., acetylpiperazine). However, there seems to be steric limitations due to a sudden

decrease in CB₂ binding affinity caused by extending the *N*-substituted alkyl chain from 2C to 3C. Moreover, large *ortho* substituents on the phenyl ring are beneficial for CB₂ binding affinities. The introduction of a methyl group at the C5 position seems to significantly improve the CB₂ binding affinities of the molecules (Figure 5). It is noteworthy that the two most potent compounds **47** and **48** ($K_i = 27.5$ nM and 23 nM, respectively) manifest CB₂ binding affinities twice as potent as the reference compound **3** ($K_i = 51$ nM). This result implies that oxazolo[5,4-*d*]pyrimidine-based compounds can act as effective bioisosteres of purines in terms of CB₂ binding. Additionally, compounds **47** (SI > 36) and **48** (SI = 12.7) are selective CB₂ ligands over CB₁ receptors.

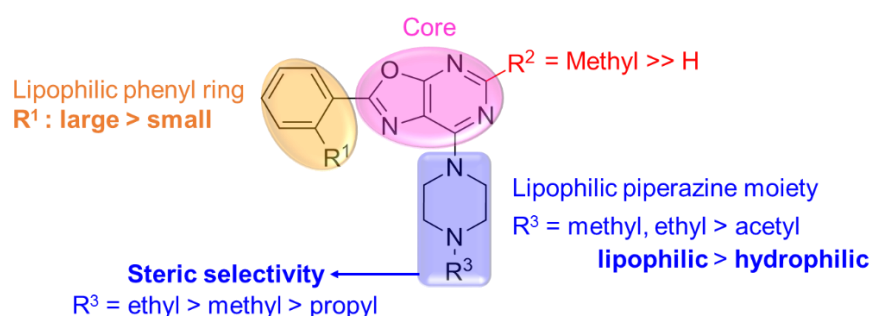


Figure 5. Structure-affinity relationships of oxazolo[5,4-*d*]pyrimidine-based CB₂ ligands.

The cytotoxicity of these compounds was determined at 10 μM using a cell proliferation assay on Chinese hamster ovary cells wild type (CHO-WT), Chinese hamster ovary cells expressing CB₂ receptors (CHO-CB₂), and human colorectal adenocarcinoma cells HT29. This test is based on a colorimetric method, which measures the activity of cellular enzymes that reduce the tetrazolium dye (MTS, yellow) to its insoluble formazan, giving a purple color. This assay measures cellular

metabolic activity via NADPH-dependent cellular oxidoreductase enzymes and reflects, under defined conditions, the number of viable cells [5, 42]. No significant cytotoxicity over 72h was observed for these new compounds.

2.3. Cyclic adenosine monophosphate (cAMP) assays

Eight compounds displaying potent CB₂ affinity were further studied for their functionality by cAMP assays in CHO-CB₂ cells. Cells were treated with forskolin (3 μ M) in order to activate adenylyl cyclase and thereby producing cAMP [43-45]. CB₂ ligands can be classified as agonists, neutral antagonists, or inverse agonists regarding to their functional effects on cAMP formation. Agonists inhibit forskolin-induced cAMP production, whereas inverse agonists promote cAMP production. On the contrary, neutral antagonists do not significantly alter cAMP levels [46, 47]. As shown in Table 2, the maximum efficacy (E_{\max}) of a compound represents the maximum response at 1 μ M and is expressed as the percentage of forskolin-induced cAMP production [5]. Despite their potent binding affinities toward CB₂ receptors, these compounds did not significantly affect CB₂-mediated regulation of cAMP accumulation (E_{\max} values range from 88% to 131%), compared with forskolin (100%). Data in Figure 6 further demonstrated that compounds **47** and **48** showed competitive antagonist properties. These two compounds remarkably antagonized CB₂ agonist (0.1 μ M WIN 55,212-2)-induced inhibition of cAMP formation at 1 μ M and almost completely restored forskolin-induced cAMP levels at 10 μ M. IC₅₀ values of these two compounds are 0.93 μ M (**47**) and 0.12 μ M (**48**), respectively.

Interestingly, although replacement of the purine core by oxazolo[5,4-*d*]pyrimidine does not alter the binding affinity of the molecule toward CB₂, it appears to alter the biological response from a CB₂ agonist to competitive neutral antagonist.

Table 2. Maximum efficacy (E_{\max}) of compounds **36-38**, **40-42**, **47-48**, and reference compounds **3**, **4**, WIN 55,212-2 at 1 μ M.

Compounds	CB ₂ cAMP assays E_{\max} (%) ^a
36	129 \pm 3
37	117 \pm 15
38	107 \pm 16
40	131 \pm 13
41	120 \pm 8
42	117 \pm 7
47	94 \pm 2
48	88 \pm 7
forskolin	100
3 (agonist)	71 \pm 3
4 (inverse agonist)	863 \pm 28
WIN55212-2 (agonist)	64 \pm 12

^a Data represent the mean \pm SEM of at least three independent experiments performed in duplicate or triplicate. E_{\max} values are expressed as the percentage of forskolin-induced cAMP production. Forskolin (3 μ M) effect was set to 100%.

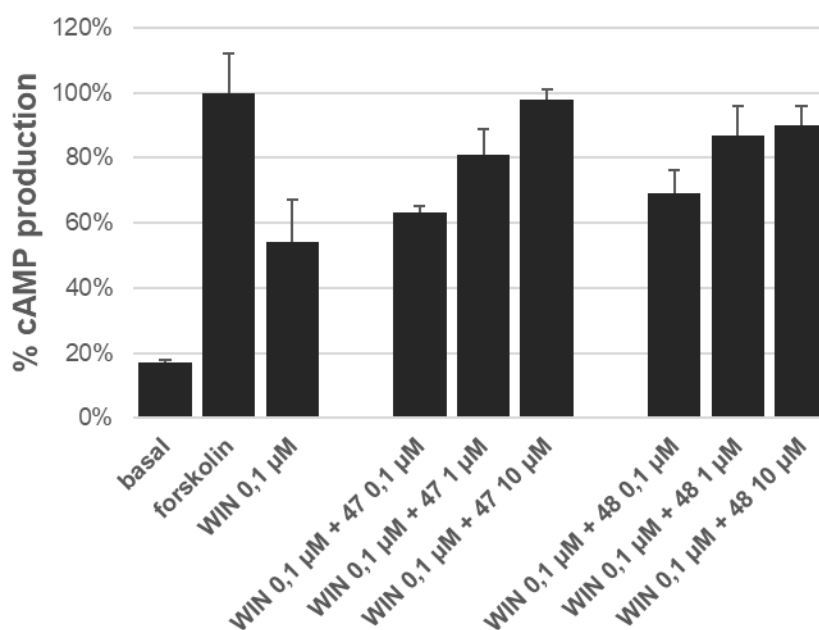


Figure 6. Inhibition of forskolin-stimulated cAMP formation by WIN 55,212-2 (0.1 μ M) and antagonism by **47** and **48** (0.1-10 μ M) in CB₂-CHO cells. Forskolin (3 μ M) effect was set to 100%.

Data represent the mean \pm SEM of at least three experiments performed in duplicate.

2.4. Metabolic stability in mice liver microsomes

These eight potent CB₂ ligands have also been examined for their in vitro metabolic stability in the presence of mice liver microsomes (Figure 7, Table 3). It was found that almost all of compounds **36**, **37**, **40** and **41** were rapidly eliminated under 5 min ($t_{1/2} \approx 1$ min). Compounds **47** and **48** showed lower intrinsic clearance ($t_{1/2} = 4.1$ and 8.1 min respectively) than compounds **40** and **41** ($t_{1/2} = 1.3$ and 1.2 min respectively). This observation implies that a C5-substituted methyl group confers higher metabolic stability to the molecules[48], although the clearance values are still rather high. In addition, the replacement of an *N*-alkyl group by an *N*-acetyl group on the piperazine moiety (**38** and **42**, $t_{1/2} = 12.5$ and 12.2 min respectively) has been deemed to endow the molecules with significantly improved metabolic stability. Although these eight newly synthesized compounds are less metabolically stable than compounds **3** in mice liver microsomes, the study of their structure-stability relationships may be helpful to further improve the metabolic stability of oxazolo[5,4-*d*]pyrimidine derivatives.

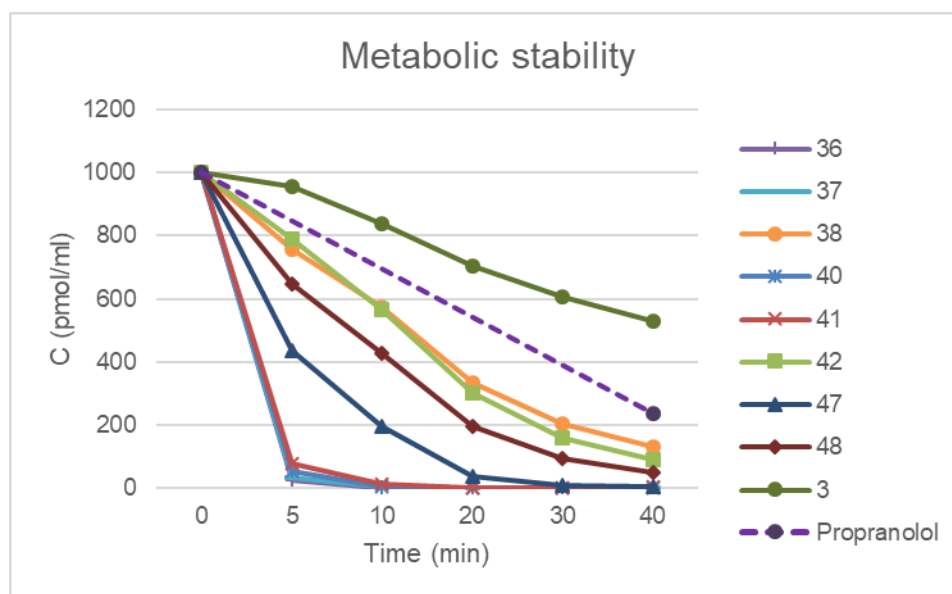


Figure 7. Metabolic stability of compounds **36-38**, **40-42**, **47**, **48**, **3**, and propranolol.

Table 3. Cl_{int} and $t_{1/2}$ of compounds **36-38**, **40-42**, **47**, **48**, and **3** in the presence of mice liver microsomes.

Compounds	Cl_{int} ($\mu\text{L}/\text{min}/\text{mg}$) ^a	$t_{1/2}$ (min)
36	1257	0.9
37	1236	1.0
38	174	12.5
40	1178	1.3
41	1101	1.2
42	194	12.2
47	502	4.1
48	263	8.1
3	54	44

^a Cl_{int} = dose/AUC, expressed as $\mu\text{L}/\text{min}/\text{mg}$ proteins.

3. Conclusion and perspective

A novel series of oxazolo[5,4-*d*]pyrimidines has been synthesized. Some of them display potent affinity toward CB₂ at nanomolar concentrations and significant selectivity over CB₁. Especially, compounds **47** and **48** have been identified as competitive neutral antagonists. Our studies indicate that oxazolo[5,4-*d*]pyrimidine-based compounds can act as effective bioisosteres of

purines in CB₂ binding. Interestingly, such a replacement changes the biological response from a CB₂ agonist to a competitive neutral antagonist.

There are many reports on CB₂ agonists and inverse agonists but few in the case of neutral antagonists. During the last few years, several clinical trials of CB₂ agonists have been launched. The therapeutic potential of CB₂ inverse agonists has also progressed. To the best of our knowledge, research on neutral antagonists is rare. Indeed, the first reported antagonist SR144528 was then identified to behave as an inverse agonist [16, 49]. Some purported “antagonists” are actually inverse agonists [50-54]. The real first reported CB₂ neutral antagonist may be WIN55212-3 (Figure 8), an enantiomer of WIN55212-2, but it showed weak affinity toward CB₂ ($K_i > 1 \mu\text{M}$). Another reported CB₂ neutral antagonist is XIE35-1001 (Figure 8) [55]. However, data in a cAMP assay or [³⁵S]-GTP γ S assay are missing. Recently, Bertini and coworkers described a non-competitive CB₂ neutral antagonist (**49**, Figure 8) [56], which significantly reversed CB₂ agonist (1 nM HU-210) responses at 10 μM but did not alter maximal responses of the agonist (0.1 μM HU-210) even at 20 μM in a [³⁵S]-GTP γ S assay. On the contrary, our two neutral antagonists **47** and **48** remarkably reversed CB₂ agonist (0.1 μM WIN 55,212-2)-induced inhibition of cAMP formation at 1 μM . This observation makes compounds **47** and **48** useful tools for studying the function of CB₂ receptors at the cellular level.

In this context, we identified that oxazolo[5,4-*d*]pyrimidine could be a useful scaffold for the development of new selective neutral antagonists toward CB₂.

Moreover, although CB₂ inverse antagonists have shown therapeutic potential in bone-dependent disorders and inflammation through somehow modulating the migration of immune cells, relevant mechanisms remain unclear. The discovery of new CB₂ neutral antagonists with high affinity might be helpful for elucidating how CB₂ inverse agonists regulate bone proliferation and the migration of immune cells. Notably, compounds **47** and **48**, which display nanomolar potency and behave as competitive neutral antagonists toward CB₂, may serve as lead compounds for further studies. Furthermore, we will continue the structural optimization of these oxazolepyrimidines to improve their metabolic stability so that they can be suitable for systemic applications.

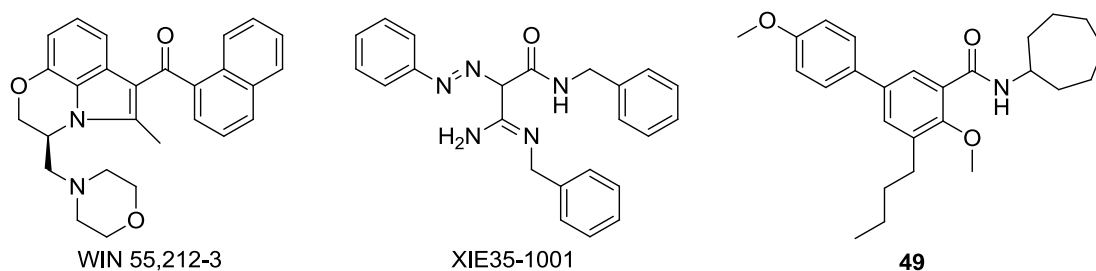


Figure 8. Structures of representative CB₂ neutral antagonists.

4. Experimental section

4.1. Chemistry

4.1.1. General procedures

All reactions involving air or moisture-sensitive compounds were performed under argon atmosphere. Solvents and reagents were purchased from commercial suppliers and were used without further purification. The thin layer chromatography

was performed using silica gel plates (Polygram Sil G/UV254, thickness: 0.2 mm, ref. 805023 Macherey Nagel). The revelation was made under UV (253 and 366 nm). The purifications by column chromatography were performed using silica gel 60, granulometry 40-63 μm (ref. 9385.5000 Merck). The microwave reactions were performed using a CEM DiscoverSP. Melting points were recorded on a Büchi melting point apparatus and are uncorrected. They are expressed in degrees Celsius. NMR spectra were recorded at 300 (^1H) and 75 (^{13}C) MHz on a Bruker AC300P Fourier transform spectrometer. Chemical shifts (δ) are reported relative to tetramethylsilane (TMS) as the internal standards in the NMR laboratory of Physical Chemistry of University Lille 2. Spectra were recorded at ambient temperature. Each signal is identified by its chemical shift δ expressed in parts per million (ppm). Coupling constant (J) is reported in Hertz (Hz). All target compounds were characterized by LC-MS and HRMS. The high performance liquid chromatograph (ODS column, mobile phase: $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{HCOOH}$ using gradient method) is coupled to a UV detector and a type of APCI⁺ (atmospheric pressure chemical ionization) mass detector. HRMS analyzes were performed under ESI (electrospray ionization) using a TOF analyzer in V mode with a mass resolution of 9000. The spectra were recorded at the Centre Universitaire des Mesures Analytique of University Lille 2 using Thermo Electron Surveyor MSQ mass spectrometer. The purity of all compounds was determined by HPLC using a Chromazing column (4.6 x 150 mm, 5 μm , 100 Å, mobile phase: $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{HCOOH}$ = 100/25/1) and a WATERS 600 pump chromatograph equipped with a WATERS 2487 dual absorption

wavelength UV detector ($\lambda = 254$ nm and 366 nm). Retention time was obtained with flow rates of 1 mL/min. The acquisition time is 20 min.

4.1.2. Synthesis of 5-amino-2-(2-substituted phenyl)oxazole-4-carbonitriles (**11-14**)

A solution of benzoyl chloride or ortho halogenated benzoyl chloride (4.8 mmol, 1.2 equiv) and aminomalononitrile p-toluenesulfonate (4 mmol, 1 equiv) in 15 mL NMP was stirred at room temperature for 10 min, and then heated by microwave irradiation at 80 °C for 25 min. At the end of the reaction, after cooling, the solution was diluted with 100 mL saturated brine and neutralized (pH ~8) to give a white precipitate, which was further recrystallized in CH₂Cl₂ to afford compounds **11-14**. These compounds were used for the next reaction.

4.1.3. Synthesis of 2-(2-chlorophenyl)oxazolo[5,4-*d*]pyrimidin-7-amine (**15**)

A solution of compound **13** (2.5 mmol, 1 equiv) and formamidine acetate (3 mmol, 1.2 equiv) in 15 mL NMP was stirred and heated at 100 °C for 3 h. At the end of the reaction, after cooling, the solution was diluted with 100 mL saturated brine to give a white precipitate, which was recrystallized in CH₂Cl₂ to afford compound **15**. This compound was used for the next reaction.

4.1.4. Synthesis of 2-(2-chlorophenyl)-*N*-alkyloxazolo[5,4-*d*]pyrimidin-7-amines (**16-20**)

To a solution of compound **15** (0.25 mmol, 0.1 equiv) and brominated derivative (0.2 mmol, 0.8 equiv) in 15 mL DMF, cooled to 0 °C, 15 wt. % KHMDS (0.3 mmol,

1.2 equiv) solution in toluene was added dropwise. Afterwards, the mixture solution was recovered to room temperature and stirred for 3 h. At the end of the reaction, the solution was diluted with 100 mL saturated brine, and then extracted with 80 mL ethyl acetate. The organic phase was washed twice with 50 mL distilled water and dried over MgSO₄. The solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography using CH₂Cl₂/ethyl acetate (8: 2 v/v) as eluent to give compounds **16-20**.

4.1.4.1. *N*-Butyl-2-(2-chlorophenyl)oxazolo[5,4-*d*]pyrimidin-7-amine (**17**)

The title compound was obtained as a white solid, mp 104-105 °C, purity (HPLC): 98.8%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.46 – 8.19 (m, 2H), 8.09 (d, *J* = 7.8 Hz, 1H), 7.72 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.70 – 7.52 (m, 2H), 3.90 – 3.71 (m, 0.5H), 3.62 – 3.41 (m, 1.5H), 1.73 – 1.50 (m, 2H), 1.48 – 1.27 (m, 2H), 0.92 (t, *J* = 7.3 Hz, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 164.1 (C), 156.2 (C), 155.5 (C), 155.2 (CH_A), 154.8 (CH_B), 133.3 (CH), 132.4 (C), 132.2 (CH), 131.7 (CH), 128.2 (CH), 125.8 (C), 116.1 (C), 42.7 (CH_{2B}), 40.3 (CH_{2A}), 32.5 (CH_{2B}), 31.4 (CH_{2A}), 20.1 (CH₂), 14.2 (CH₃). HRMS (ESI) calcd. for C₁₅H₁₅ON₄Cl ([M+H]⁺) 303.10072, found 303.10004.

4.1.4.2. 2-(2-Chlorophenyl)-*N*-(2-methoxyethyl)oxazolo[5,4-*d*]pyrimidin-7-amine (**18**)

The title compound was obtained as a white solid, mp 124-125 °C, purity (HPLC): 95.1%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.43 – 8.20 (m, 2H), 8.10 (dd, *J* =

7.6, 2.0 Hz, 1H), 7.72 (dd, $J = 7.8, 1.6$ Hz, 1H), 7.70 – 7.52 (m, 2H), 4.09 – 3.88 (m, 0.5H), 3.76 – 3.64 (m, 1.5H), 3.63 – 3.51 (m, 2H), 3.28 (s, 3H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 164.1 (C), 156.3 (C), 155.5 (C), 155.1 (CH), 133.3 (CH), 132.4 (C), 132.2 (CH), 131.7 (CH), 128.2 (CH), 125.7 (C), 116.3 (C), 71.4 (CH_{2B}), 70.5 (CH_{2A}), 58.4 (CH₃), 42.6 (CH_{2B}), 40.1 (CH_{2A}). HRMS (ESI) calcd. for C₁₄H₁₃O₂N₄Cl ([M+H]⁺) 305.07998, found 305.07927.

4.1.4.3. 2-(2-Chlorophenyl)-*N*-pentyloxazolo[5,4-*d*]pyrimidin-7-amine (**19**)

The title compound was obtained as a white solid, mp 122-123 °C, purity (HPLC): 98.4%. ^1H NMR (300 MHz, DMSO- d_6) δ 8.43 – 8.19 (m, 2H), 8.08 (dd, $J = 7.5, 1.9$ Hz, 1H), 7.70 (dd, $J = 7.9, 1.5$ Hz, 1H), 7.68 – 7.50 (m, 2H), 3.86 – 3.68 (m, 0.5H), 3.58 – 3.41 (m, 1.5H), 1.72 – 1.49 (m, 2H), 1.38 – 1.25 (m, 4H), 0.86 (t, $J = 6.7$ Hz, 3H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 164.1 (C), 156.2 (C), 155.5 (C), 155.2 (CH_A), 154.8 (CH_B), 133.3 (CH), 132.4 (C), 132.2 (CH), 131.7 (CH), 128.2 (CH), 125.8 (C), 116.1 (C), 43.0 (CH_{2B}), 40.8 (CH_{2A}), 30.0 (CH_{2B}), 29.0 (CH_{2A} × 2), 22.4 (CH₂), 14.4 (CH₃). HRMS (ESI) calcd. for C₁₆H₁₇ON₄Cl ([M+H]⁺) 317.11637, found 317.11586.

4.1.4.4. 2-(2-Chlorophenyl)-*N*-(cyclohexylmethyl)oxazolo[5,4-*d*]pyrimidin-7-amine (**20**)

The title compound was obtained as a solid, mp 140-141 °C, purity (HPLC): 97.6%. ^1H NMR (300 MHz, DMSO- d_6) δ 8.45 – 8.20 (m, 2H), 8.08 (d, $J = 7.7$ Hz, 1H), 7.76 – 7.66 (m, 1H), 7.69 – 7.51 (m, 2H), 3.70 – 3.57 (m, 0.5H), 3.36 (t, $J = 6.4$

Hz, 1.5H), 1.83 – 1.55 (m, 6H), 1.28 – 1.11 (m, 3H), 1.06 – 0.86 (m, 2H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 164.1 (C), 156.2 (C), 155.7 (C), 155.1 (CH_A), 154.8 (CH_B), 133.3 (CH), 132.4 (C), 132.3 (CH), 131.7 (CH), 128.2 (CH), 125.8 (C), 116.1 (C), 48.9 ($\text{CH}_{2\text{B}}$), 46.7 ($\text{CH}_{2\text{A}}$), 38.6 (CH_B), 37.6 (CH_A), 30.9 ($\text{CH}_2 \times 2$), 26.6 (CH_2), 25.8 ($\text{CH}_2 \times 2$). HRMS (ESI) calcd. for $\text{C}_{18}\text{H}_{20}\text{ON}_4\text{Cl}$ ($[\text{M}+\text{H}]^+$) 343.13202, found 343.13139.

4.1.5. Synthesis of 2-(2-chlorophenyl)-*N*-(piperidin-4-ylmethyl)oxazolo[5,4-*d*]pyrimidin-7-amine (**21**)

To a solution of compound **16** (0.2 mmol, 1 equiv) in 50 mL CH_2Cl_2 was added TFA (2 mmol, 10 equiv). The mixture was stirred at room temperature for 3 h. At the end of the reaction, the solvent was removed under reduced pressure and the residue was dissolved in water and the solution was neutralised to pH 9 using 30% NaOH, then the solution was extracted with ethyl acetate. The organic layer was washed with brine and distilled water successively, dried over MgSO_4 and evaporated under reduced pressure to give compound **21**. The title compound was obtained as a white solid, mp > 270 °C, purity (HPLC): 96.8%. ^1H NMR (300 MHz, DMSO- d_6) δ 8.53 – 8.19 (m, 2H), 8.09 (d, $J = 7.6$ Hz, 1H), 7.71 (dd, $J = 7.8, 1.6$ Hz, 1H), 7.70 – 7.51 (m, 2H), 3.71 – 3.61 (m, 0.5H), 3.44 – 3.33 (m, 1.5H), 3.00 – 2.84 (m, 2H), 2.46 – 2.31 (m, 2H), 1.88 – 1.52 (m, 3H), 1.20 – 0.92 (m, 2H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 164.1 (C), 156.2 (C), 155.7 (C), 155.1 (CH_A), 154.8 (CH_B), 133.3 (CH), 132.4 (C), 132.2 (CH), 131.7 (CH), 128.2 (CH), 125.8 (C), 116.1 (C), 48.9 ($\text{CH}_{2\text{B}}$), 46.7 ($\text{CH}_{2\text{A}}$),

46.3 ($\text{CH}_2 \times 2$), 37.7 (CH_B), 36.6 (CH_A), 31.5 (CH_2), 31.2 (CH_2). HRMS (ESI) calcd. for $\text{C}_{17}\text{H}_{19}\text{ON}_5\text{Cl}$ ($[\text{M}+\text{H}]^+$) 344.12726, found 344.12650.

4.1.6. Synthesis of 2-(2-substituted phenyl)oxazolo[5,4-*d*]pyrimidin-7-ols (**22-25**)

To a solution of compound **11**, **12**, **13**, or **14** (1 mmol) in 20 mL formic acid was refluxed overnight. At the end of the reaction, after cooling, formic acid was removed under reduced pressure. The residue was purified by silica gel column chromatography using CH_2Cl_2 /ethyl acetate (6: 4 v/v) as eluent to give compound **22**, **23**, **24**, or **25**, respectively.

4.1.7. Synthesis of 7-chloro-2-(2-substituted phenyl)oxazolo[5,4-*d*]pyrimidines (**26-29**)

To a solution of compound **22**, **23**, **24**, or **25** (0.5 mmol, 1 equiv) in 30 mL toluene were added DIPEA (0.75 mmol, 1.5 equiv) and POCl_3 (1.5 mmol, 3 equiv) successively at 0 °C. The mixture was stirred for 2 h. At the end of the reaction, the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using CH_2Cl_2 /ethyl acetate (95: 5 v/v) as eluent to give compound **26**, **27**, **28**, or **29**, respectively.

4.1.8. Synthesis of 2-(2-substituted phenyl)-7-(4-substituted piperazinyl or morpholine)oxazolo[5,4-*d*]pyrimidines (**30**, **32-42**)

To a solution of compound **26**, **27**, **28**, or **29** (0.15 mmol, 1 equiv) in 15 mL DMF were added Cs_2CO_3 (0.3 mmol, 2 equiv) and piperazine derivative/morpholine

(0.18 mmol, 1.2 equiv). The mixture was stirred and heated at 100 °C for 30 min. At the end of the reaction, the solution was diluted with 50 mL saturated brine, and then extracted twice with 30 mL ethyl acetate. The organic phase was washed twice with 50 mL distilled water and dried over MgSO₄. The solvent was evaporated under reduced pressure to give pure target compound directly or crude compound, which was purified by silica gel column chromatography using ethyl acetate/methanol (98: 2 v/v) as eluent.

4.1.8.1. 7-(4-Methylpiperazin-1-yl)-2-phenyloxazolo[5,4-*d*]pyrimidine (**32**)

The title compound was obtained as a yellow solid, mp 136-137 °C, purity (HPLC): 99.3%. ¹H NMR (300 MHz, CDCl₃) δ 8.36 (s, 1H), 8.24 – 8.13 (m, 2H), 7.56 – 7.46 (m, 3H), 4.55 – 4.06 (m, 4H), 2.64 – 2.52 (m, 4H), 2.38 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 165.5 (C), 157.5 (C), 154.1 (C), 153.7 (CH), 131.6 (CH), 128.9 (CH × 2), 127.3 (CH × 2), 126.5 (C), 116.9 (C), 55.0 (CH₂ × 2), 46.2 (CH₃), 45.1 (CH₂ × 2). HRMS (ESI) calcd. for C₁₆H₁₈ON₅ ([M+H]⁺) 296.15059, found 296.14967.

4.1.8.2. 7-(4-Ethylpiperazin-1-yl)-2-phenyloxazolo[5,4-*d*]pyrimidine (**33**)

The title compound was obtained as a yellow solid, mp 120-121 °C, purity (HPLC): 99.3%. ¹H NMR (300 MHz, CDCl₃) δ 8.36 (s, 1H), 8.25 – 8.12 (m, 2H), 7.56 – 7.47 (m, 3H), 4.57 – 4.00 (m, 4H), 2.70 – 2.57 (m, 4H), 2.50 (q, *J* = 7.1 Hz, 2H), 1.15 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 165.5 (C), 157.5 (C), 154.1 (C), 153.7 (CH), 131.5 (CH), 128.9 (CH × 2), 127.3 (CH × 2), 126.5 (C), 116.8

(C), 52.8 (CH₂ × 2), 52.4 (CH₂), 44.9 (CH₂ × 2), 11.9 (CH₃). HRMS (ESI) calcd. for C₁₇H₂₀ON₅ ([M+H]⁺) 310.16624, found 310.16501.

4.1.8.3. 2-(2-Fluorophenyl)-7-(4-methylpiperazin-1-yl)oxazolo[5,4-*d*]pyrimidine (**34**)

The title compound was obtained as a yellow solid, mp 142-143 °C, purity (HPLC): 98.1%. ¹H NMR (300 MHz, CDCl₃) δ 8.38 (s, 1H), 8.19 – 8.11 (m, 1H), 7.56 – 7.46 (m, 1H), 7.33 – 7.27 (m, 1H), 7.25 – 7.19 (m, 1H), 4.54 – 4.09 (m, 4H), 2.57 (t, *J* = 5.0 Hz, 4H), 2.37 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 165.3 (C), 160.67 (C, d, *J* = 259.8 Hz), 154.2 (C), 154.0 (CH), 153.8 (C, d, *J* = 5.7 Hz), 133.1 (CH, d, *J* = 8.5 Hz), 130.1 (CH), 124.5 (CH, d, *J* = 3.8 Hz), 117.1 (CH, d, *J* = 21.0 Hz), 116.6 (C), 115.0 (C, d, *J* = 10.6 Hz), 55.0 (CH₂ × 2), 46.2 (CH₃), 45.1 (CH₂ × 2). HRMS (ESI) calcd. for C₁₆H₁₇ON₅F ([M+H]⁺) 314.14116, found 314.14037.

4.1.8.4. 7-(4-Ethylpiperazin-1-yl)-2-(2-fluorophenyl)oxazolo[5,4-*d*]pyrimidine (**35**)

The title compound was obtained as a yellow solid, mp 127-128 °C, purity (HPLC): 99.4%. ¹H NMR (300 MHz, CDCl₃) δ 8.38 (s, 1H), 8.19 – 8.11 (m, 1H), 7.56 – 7.46 (m, 1H), 7.33 – 7.27 (m, 1H), 7.25 – 7.19 (m, 1H), 4.54 – 4.07 (m, 4H), 2.61 (t, *J* = 5.1 Hz, 4H), 2.50 (q, *J* = 7.2 Hz, 2H), 1.15 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 165.3 (C), 160.7 (C, d, *J* = 259.9 Hz), 154.1 (C), 154.0 (CH), 153.8 (C, d, *J* = 5.6 Hz), 133.1 (CH, d, *J* = 8.5 Hz), 130.1 (CH), 124.5 (CH, d, *J* = 3.9 Hz), 117.1 (CH, d, *J* = 21.0 Hz), 116.6 (C), 115.0 (C, d, *J* = 10.4 Hz), 52.8 (CH₂ × 2), 52.4 (CH₂), 45.1 (CH₂ × 2), 11.9 (CH₃). HRMS (ESI) calcd. for C₁₇H₁₉ON₅F ([M+H]⁺) 328.15681, found 328.15533.

4.1.8.5.

7-(4-Methylpiperazin-1-yl)-2-(2-(trifluoromethyl)phenyl)oxazolo[5,4-*d*]pyrimidine

(36)

The title compound was obtained as a yellow solid, mp 137-138 °C, purity (HPLC): 99.3%. ¹H NMR (300 MHz, CDCl₃) δ 8.39 (s, 1H), 8.19 – 8.09 (m, 1H), 7.93 – 7.83 (m, 1H), 7.77 – 7.59 (m, 2H), 4.57 – 3.99 (m, 4H), 2.58 (t, *J* = 5.0 Hz, 4H), 2.37 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 165.6 (C), 154.7 (C), 154.3 (CH, C), 132.0 (CH), 131.6 (CH), 131.1 (CH), 129.0 (C, q, *J* = 32.2 Hz), 127.4 (CH, q, *J* = 5.8 Hz), 125.3 (C), 123.3 (CF₃, q, *J* = 273.7 Hz), 116.6 (C), 54.9 (CH₂ × 2), 46.1 (CH₃), 45.1 (CH₂ × 2). HRMS (ESI) calcd. for C₁₇H₁₇ON₅F₃ ([M+H]⁺) 364.13797, found 364.13656.

4.1.8.6.

7-(4-Ethylpiperazin-1-yl)-2-(2-(trifluoromethyl)phenyl)oxazolo[5,4-*d*]pyrimidine **(37)**

The title compound was obtained as a yellow solid, mp 104-105 °C, purity (HPLC): 98.1%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.39 (s, 1H), 8.18 (d, *J* = 7.3 Hz, 1H), 8.02 (d, *J* = 7.3 Hz, 1H), 7.96 – 7.80 (m, 2H), 4.51 – 3.86 (m, 4H), 2.59 – 2.50 (m, 4H), 2.38 (q, *J* = 7.1 Hz, 2H), 1.03 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 165.6 (C), 154.7 (C), 154.3 (CH, C), 132.0 (CH), 131.6 (CH), 131.1 (CH), 129.0 (C, q, *J* = 32.2 Hz), 127.4 (CH, q, *J* = 5.8 Hz), 125.3 (C), 123.3 (CF₃, q, *J* = 271.9 Hz), 116.6 (C), 52.7 (CH₂ × 2), 52.4 (CH₂), 45.2 (CH₂ × 2), 11.9 (CH₃). HRMS (ESI) calcd. for C₁₈H₁₉ON₅F₃ ([M+H]⁺) 378.15362, found 378.15199.

4.1.8.7.

1-(4-(2-(2-(Trifluoromethyl)phenyl)oxazolo[5,4-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone (**38**)

The title compound was obtained as a yellow solid, mp 145-146 °C, purity (HPLC): 96.3%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.42 (s, 1H), 8.19 (d, *J* = 6.7 Hz, 1H), 8.07 – 7.98 (m, 1H), 7.96 – 7.83 (m, 2H), 4.45 – 3.89 (m, 4H), 3.71 – 3.55 (m, 4H), 2.07 (s, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 169.0 (C=O), 165.7 (C), 155.1 (C), 154.6 (CH), 154.2 (C), 133.6 (CH), 132.7 (CH), 132.6 (CH), 127.7 (C, *q*, *J* = 31.9 Hz), 127.9 (CH, *q*, *J* = 5.5 Hz), 124.7 (C), 123.9 (CF₃, *q*, *J* = 273.3 Hz), 116.2 (C), 45.7 (CH₂), 44.9 (CH₂ × 2), 41.0 (CH₂), 21.7 (CH₃). HRMS (ESI) calcd. for C₁₈H₁₇O₂N₅F₃ ([M+H]⁺) 392.13289, found 392.13206.

4.1.8.8. 2-(2-Chlorophenyl)-7-morpholinoxazolo[5,4-*d*]pyrimidine (**39**)

The title compound was obtained as a yellow solid, mp 155-156 °C, purity (HPLC): 98.2%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.40 (s, 1H), 8.12 (dd, *J* = 7.6, 1.9 Hz, 1H), 7.71 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.67 – 7.53 (m, 2H), 4.50 – 3.88 (m, 4H), 3.76 (t, *J* = 4.8 Hz, 4H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 165.4 (C), 155.2 (C), 154.5 (CH), 154.2 (C), 133.5 (CH), 132.5 (C), 132.1 (CH), 131.9 (CH), 128.3 (CH), 125.1 (C), 116.1 (C), 66.5 (CH₂ × 2), 45.8 (CH₂ × 2). HRMS (ESI) calcd. for C₁₅H₁₄O₂N₄Cl ([M+H]⁺) 317.07998, found 317.07944.

4.1.8.9. 2-(2-Chlorophenyl)-7-(4-methylpiperazin-1-yl)oxazolo[5,4-*d*]pyrimidine (**40**)

The title compound was obtained as a yellow solid, mp 135-136 °C, purity (HPLC): 99.9%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.37 (s, 1H), 8.14 – 8.07 (m, 1H), 7.74 – 7.67 (m, 1H), 7.66 – 7.53 (m, 2H), 4.47 – 3.87 (m, 4H), 2.45 (t, *J* = 5.0 Hz, 4H), 2.22 (s, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 165.4 (C), 155.0 (C), 154.5 (CH), 154.1 (C), 133.4 (CH), 132.4 (C), 132.1 (CH), 131.8 (CH), 128.3 (CH), 125.1 (C), 116.0 (C), 54.8 (CH₂ × 2), 46.1 (CH₃), 45.1 (CH₂ × 2). HRMS (ESI) calcd. for C₁₆H₁₇ON₅Cl ([M+H]⁺) 330.11161, found 330.11082.

4.1.8.10. 2-(2-Chlorophenyl)-7-(4-ethylpiperazin-1-yl)oxazolo[5,4-*d*]pyrimidine (**41**)

The title compound was obtained as a yellow solid, mp 115-116 °C, purity (HPLC): 99.0%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.38 (s, 1H), 8.14 – 8.08 (m, 1H), 7.74 – 7.68 (m, 1H), 7.67 – 7.54 (m, 2H), 4.52 – 3.89 (m, 4H), 2.75 – 2.50 (m, 4H), 2.47 – 2.32 (m, 2H), 1.05 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 165.4 (C), 155.1 (C), 154.5 (CH), 154.1 (C), 133.4 (CH), 132.5 (C), 132.1 (CH), 131.9 (CH), 128.3 (CH), 125.1 (C), 116.0 (C), 52.5 (CH₂), 51.9 (CH₂ × 2), 44.8 (CH₂ × 2), 12.2 (CH₃). HRMS (ESI) calcd. for C₁₇H₁₉ON₅Cl ([M+H]⁺) 344.12726, found 344.12641.

4.1.8.11.

1-(4-(2-(2-Chlorophenyl)oxazolo[5,4-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone (**42**)

The title compound was obtained as a yellow solid, mp 189-190 °C, purity (HPLC): 97.3%. ¹H NMR (300 MHz, CDCl₃) δ ¹H NMR 8.43 (s, 1H), 8.17 – 8.07 (m, 1H), 7.62 – 7.53 (m, 1H), 7.53 – 7.37 (m, 2H), 4.49 – 4.12 (m, 4H), 3.80 (t, *J* = 5.4 Hz, 2H), 3.65 (t, *J* = 5.2 Hz, 2H), 2.19 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 169.3 (C=O),

165.3 (C), 155.6 (C), 154.1 (C, CH), 133.5 (C), 132.2 (CH), 131.7 (CH), 131.3 (CH), 127.1 (CH), 125.0 (C), 116.7 (C), 46.2 (CH₂), 45.1 (CH₂ × 2), 41.4 (CH₂), 21.5 (CH₃). HRMS (ESI) calcd. for C₁₇H₁₇O₂N₅Cl ([M+H]⁺) 358.10653, found 358.10581.

4.1.9. Synthesis of 2-(2-chlorophenyl)-7-(piperazin-1-yl)oxazolo[5,4-*d*]pyrimidine (**31**)

The synthesis of **31** started from compound **30**. The synthetic procedure is the same as in part 4.1.5. It was used for the next step directly.

4.1.10. Synthesis of 2-(2-chlorophenyl)-7-(4-propylpiperazin-1-yl)oxazolo[5,4-*d*]pyrimidine (**43**)

To a solution of compound **31** (0.15 mmol, 1 equiv) in 15 mL DMF were added Cs₂CO₃ (0.3 mmol, 2 equiv) and bromopropane (0.18 mmol, 1.2 equiv). The mixture was stirred and heated at 100 °C for 30 min. At the end of the reaction, after cooling, the solution was diluted with 50 mL saturated brine, and then extracted twice with 30 mL ethyl acetate. The organic phase was washed twice with 50 mL distilled water and dried over MgSO₄. The solvent was evaporated under reduced pressure to give target compound **43**. The title compound is yellow solid, mp 136-137 °C, purity (HPLC): 95.5%. ¹H NMR (300 MHz, CDCl₃) δ 8.38 (s, 1H), 8.14 – 8.07 (m, 1H), 7.59 – 7.53 (m, 1H), 7.48 – 7.38 (m, 2H), 4.68 – 3.89 (m, 4H), 2.68 – 2.52 (m, 4H), 2.38 (t, *J* = 7.7 Hz, 2H), 1.64 – 1.50 (m, 2H), 0.94 (t, *J* = 7.4 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 165.3 (C), 155.1 (C), 154.2 (C), 154.1 (CH), 133.4 (C), 131.9 (CH), 131.6 (CH), 131.3 (CH), 127.0 (CH), 125.3 (C), 116.5 (C), 60.6 (CH₂), 53.2 (CH₂ × 2), 45.0 (CH₂

× 2), 19.9 (CH₂), 11.9 (CH₃). HRMS (ESI) calcd. for C₁₈H₂₁ON₅Cl ([M+H]⁺) 358.14291, found 358.14179.

4.1.11. Synthesis of *N*-(2-(2-chlorophenyl)-4-cyanooxazol-5-yl)acetamide (**44**)

To solution of compound **13** (1 mmol, 1 equiv) in 30 mL NMP was added DIPEA (4 mmol, 4 equiv) and acetyl chloride (4 mmol, 4 equiv) dropwise, successively. The mixture was stirred and heated at 45 °C for 6 h. At the end of the reaction, after cooling, the solution was diluted with 200 mL saturated brine, and then extracted twice with 100 mL ethyl acetate. The organic phase was washed twice with 150 mL distilled water and dried over MgSO₄. The solvent was evaporated under reduced pressure. The crude product was used for the next step without purification.

4.1.12. Synthesis of 2-(2-chlorophenyl)-5-methyloxazolo[5,4-*d*]pyrimidin-7-ol (**45**)

A solution of crude **44** (0.5 mmol) in 30 mL 30% NaOH and 10 mL 35% H₂O₂ (aqueous solution) was refluxed for 2 h. At the end of the reaction, the solution was acidified using 3 N HCl to pH 2 and a white flocculent precipitate appeared. The mixture was extracted twice with 30 mL ethyl acetate. The organic phase was washed twice with 50 mL distilled water and dried over MgSO₄. The solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using CH₂Cl₂/ethyl acetate (6: 4 v/v) as eluent to give compound **45**.

4.1.13. Synthesis of 7-chloro-2-(2-chlorophenyl)-5-methyloxazolo[5,4-*d*]pyrimidine (**46**)

The synthesis of **46** started from compound **45**. The synthetic procedure is the same as in part 4.1.7.

4.1.14. Synthesis of 2-(2-chlorophenyl)-5-methyl-7-(4-substituted piperazin-1-yl)oxazolo [5,4-*d*]pyrimidines (**47-48**)

The synthesis of compounds **47-48** started from compound **46**. The synthetic procedure is the same as in part 4.1.8.

4.1.14.1.

2-(2-Chlorophenyl)-5-methyl-7-(4-methylpiperazin-1-yl)oxazolo[5,4-*d*]pyrimidine (**47**)

The title compound was obtained as a yellow solid, mp 151-152 °C, purity (HPLC): 99.5%. ¹H NMR (300 MHz, CDCl₃) δ 8.13 – 8.05 (m, 1H), 7.58 – 7.51 (m, 1H), 7.46 – 7.37 (m, 2H), 4.68 – 4.07 (m, 4H), 2.81 – 2.62 (m, 4H), 2.59 (s, 3H), 2.44 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 166.0 (C), 164.1 (C), 154.7 (C), 153.7 (C), 133.3 (C), 131.7 (CH), 131.6 (CH), 131.2 (CH), 127.0 (CH), 125.4 (C), 114.3 (C), 54.8 (CH₂ × 2), 45.9 (CH₃), 44.7 (CH₂ × 2), 26.1 (CH₃). HRMS (ESI) calcd. for C₁₇H₁₉ON₅Cl ([M+H]⁺) 344.12726, found 344.12607.

4.1.14.2.

2-(2-Chlorophenyl)-7-(4-ethylpiperazin-1-yl)-5-methyloxazolo[5,4-*d*]pyrimidine (**48**)

The title compound was obtained as a yellow solid, mp 139-140 °C, purity (HPLC): 99.2%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.06 (d, *J* = 7.7 Hz, 1H), 7.68 (d, *J*

= 7.9, 1.6 Hz, 1H), 7.63 – 7.51 (m, 2H), 4.43 – 3.85 (m, 4H), 3.44 – 3.38 (m, 2H), 3.30 – 3.22 (m, 2H), 2.47 (s, 3H), 2.37 (q, $J = 7.2$ Hz, 2H), 1.03 (t, $J = 7.2$ Hz, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 166.0 (C), 164.3 (C), 155.4 (C), 153.2 (C), 133.5 (C), 132.0 (CH), 131.7 (CH), 131.2 (CH), 127.1 (CH), 125.1 (C), 114.6 (C), 52.6 ($\text{CH}_2 \times 2$), 51.6 (CH_2), 42.5 ($\text{CH}_2 \times 2$), 26.1 (CH_3), 9.7 (CH_3). HRMS (ESI) calcd. for $\text{C}_{18}\text{H}_{21}\text{ON}_5\text{Cl}$ ($[\text{M}+\text{H}]^+$) 358.14291, found 358.14184.

4.2. Biological evaluation

4.2.1. Competition binding assay

Stock solutions of the compounds were prepared in DMSO and further diluted with the binding buffer to the desired concentration. Briefly, [^3H]-CP-55,940 (0.5 nM), nonselective human CB_1 and CB_2 cannabinoid receptor, were added to 6 μg of membranes from CB_1 - or CB_2 -overexpressing CHO cells in binding buffer (50 mM Tris-HCl, 5 mM MgCl_2 , 2.5 mM EDTA, 0.5 mg/mL BSA, pH 7.4). After 90 min at 30 °C, the incubation was stopped and the solutions were rapidly filtered over a UniFilter-96 GF/C glass fiber plate, presoaked in PEI (0.05%) on a Filtermate UniFilter 96-Harvester (PerkinElmer), and washed 10 times with ice-cold 50 mM Tris-HCl pH 7.4. The radioactivity on the filters was measured using a TopCount NXT microplate scintillation counter (PerkinElmer) using 30 μL of MicroScint 40 (PerkinElmer). Assays were performed at least in three independent experiments in duplicate or triplicate. The nonspecific binding was determined in the presence of 5 μM (R)-(+)-WIN 55,212-2 (Sigma).

4.2.2. Cell culture and proliferation assay

CHO-WT, CHO-CB₂, and HT29 cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂, in HAM F12 medium or DMEM-GlutaMAX medium (for HT29 cells, Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 µg/mL). In the cell proliferation assay, cells were plated in triplicate on 96-well plates (3 × 10³ cells per well) and incubated for 24 h. Cells were then incubated in culture medium that contained 10 µM test compounds. After 72 h, cell growth was estimated by the colorimetric MTS test.

4.2.3. Cell-based HTRF cAMP assay

Cellular cAMP levels were measured using reagents supplied by Cisbio International (HTRF dynamic 2 cAMP kit). Briefly, CHO-CB₂ cells were harvested and were collected by centrifugation for 5 min at 1200 rpm. The cells were then resuspended in an appropriate final volume of culture medium and incubated with the phosphodiesterase inhibitor IBMX (0.5 mM). Cells were incubated for 15 min with the compounds at room temperature in a 384-well plate (2000 cells per well) before the addition of forskolin (3 µM) for 30 min at room temperature. The dye d2-conjugated cAMP and Europium cryptate-conjugated anti-cAMP antibodies were added to the assay plate, according to manufacturer's instructions. After 1 hour incubation at room temperature, the plate was read on a Spectramax microplate reader (Molecular Devices) with excitation wavelength at 340 nm and emission wavelengths

at 665 nm and 620 nm. Assays were performed at least in three independent experiments in duplicate or triplicate.

4.2.4. Metabolic stability in mice liver microsomes

The 1 μ M compounds tested and referenced were incubated in duplicate (0.5 mL reaction volume) with male mice (CD-1) liver microsomes (20 mg/mL, BD Gentest™) at 37 °C in 50 mM phosphate buffer (5 mM MgCl₂, 1 mM NADP, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 5 mM glucose-6-phosphate, pH 7.4). For the estimation of CL_{int}: sampling of a 50 μ L aliquot at 0, 5, 10, 20, 30 and 40 min, and the reaction was stopped by 4 volumes of acetonitrile containing the internal standard (200 nM compound **40** or 200 nM compound **42**). After centrifugation at 10000 g, 10 min, 4 °C, the supernatants were kept at 4 °C to be analyzed immediately (or placed at -80 °C if the analysis was postponed). Controls (t₀ and t_{final}) in triplicate: incubation with microsomes denatured by acetonitrile containing the internal standard. Propranolol was used as a positive control. Analysis was determined by UPLC-MS/MS (Waters* Acquity I-Class / Xevo TQD) using a column (Waters* Acquity BEH C18, 50 x 2.1 mm, 1.7 μ m; mobile phase: A: 10 mM ammonium ethanoate, B: CH₃CN 0.1% HCOOH). Retention time was obtained with flow rates of 600 μ L/min. The acquisition time is 4 min.

Conflict of interest

None of the authors have a conflict of interest to declare.

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Appendix A. Supplementary data

Representative ^1H , ^{13}C , and HSQC spectra.

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